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Nature, Type of Linkage, Quantity, and Absolute Configuration of (3-Hydroxy) Fatty Acids in Lipopolysaccharides from *Bacteroides fragilis* NCTC 9343 and Related Strains

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The main fatty acids present in lipopolysaccharides from *Bacteroides fragilis* NCTC 9343 were identified as 13-methyl-tetradecanoic, D-3-hydroxypentadecanoic, D-3-hydroxyhexadecanoic, D-3-hydroxy-15-methyl-hexadecanoic, and D-3-hydroxyheptadecanoic acids. Of these, 13-methyl-tetradecanoic acid is exclusively ester bound, and 3-hydroxy-15-methyl-hexadecanoic acid is exclusively involved in amide linkage. The other 3-hydroxy fatty acids are both ester and amide bound. All 3-hydroxy fatty acids possess the D configuration, and the 3-hydroxyl group of ester-linked 3-hydroxy fatty acids is not substituted. Lipopolysaccharides of related *Bacteroides* species (*B. thetaiotaomicron, B. ovatus, B. distasonis,* and *B. vulgatus*) showed a fatty acid spectrum with both similar and distinct features compared to that of *B. fragilis* lipopolysaccharides.

Lipopolysaccharides (LPS) represent the O-antigens and endotoxins of gram-negative bacteria. Chemically, the LPS of different bacterial origins consist of a heteropolysaccharide portion and a covalently bound lipid component, termed lipid A (6). By mild acid hydrolysis these two LPS portions can be separated, and immunobiological investigations into the isolated components have revealed that the polysaccharide part determines the O-specificity and that the lipid A portion is responsible for the endotoxic properties of LPS (6).

Lipid A of most gram-negative bacteria studied consists of a β1.6-linked D-glucosamine disaccharide, which carries (substituted) phosphate groups, as well as long-chain fatty acids, among which D-3-hydroxy fatty acids predominate (6, 23). Since chemically or enzymatically deacylated endotoxin preparations exhibit diminished biological activity, it appears that the presence of fatty acids in lipid A is essential for endotoxicity (6, 20). It is not known, however, whether the quantity alone, or the nature and type of linkage of fatty acids as well, is of importance for the expression of biological activity.

The LPS of several *Bacteroides* species, including *Bacteroides fragilis* LPS, have been shown to be of low endotoxic activity (27, 28) compared to enterobacterial LPS, like that of *Salmonella* (6, 20). In view of the possible significance of fatty acids in the expression of endotoxic activity, it was of interest to analyze the fatty acid spectrum of the LPS isolated from *B. fragilis* and related species.

The present paper describes the identification of the nature, type of linkage and quantity of fatty acids, and the absolute configuration of 3-hydroxy fatty acids present in the LPS of *B. fragilis*. It will be shown that, in *B. fragilis* LPS, ester- and amide-bound D-3-hydroxy fatty acids predominate. In addition, larger amounts of iso-branched pentadecanoic acid are present in ester linkage.

MATERIALS AND METHODS

Organisms and cultivation. *B. fragilis* NCTC 9343 and *Bacteroides thetaiotaomicron* NCTC 10582 were obtained from the National Collection of Type Cultures, London. *Bacteroides ovatus* ATCC 8483, *Bacteroides distasonis* ATCC 8503, and *Bacteroides vulgatus* ATCC 8482 were obtained from the American Type Culture Collection, Rockville, Md. *B. fragilis* strain E-323 was obtained from the Institut Pasteur de Lille.

B. fragilis NCTC 9343, *B. thetaiotaomicron* NCTC 10582, *B. ovatus* ATCC 8483, *B. distasonis* ATCC 8503, and *B. vulgatus* ATCC 8482 were cultured in a 3-liter fermentor (Fl 103, Biotec, Stockholm) and grown to late logarithmic phase (16). After addition of Formalin (15), the organisms were harvested, washed twice in phosphate-buffered saline (pH 7.4), and prepared for extraction of the LPS. *B. fragilis* strain E-323 was cultured in 500-ml screw-cap bottles filled to the top with medium (9). After incubation for 2 days

(37°C), the cells were harvested by centrifugation, washed twice with phosphate-buffered saline, and stored as a paste at −25°C.

Isolation and purification of LPS. Washed organisms were suspended in distilled water to give 20 mg (dry weight)/ml and extracted by the phenol-water procedure (29). The LPS was recovered from the water phase and lyophilized. If necessary, LPS was treated with RNase as described earlier (16).

In the case of *B. fragilis* strain E-323, the cells were suspended in distilled water (100 mg [wet weight]/ml) and extracted by constant stirring with equal volumes of phenol (90%) for 60 min at 22°C. After dialysis against tap water (2 days), the water phase was centrifuged (8,000 × *g*, 30 min), and LPS was separated from the supernatant fluid by ultracentrifugation (100,000 × *g*, 90 min) and purified by treatment with RNase and DNase (10). The LPS preparation obtained was washed twice with chloroform-methanol (2:1, vol/vol).

Purification of LPS from *B. fragilis* NCTC 9343. LPS from *B. fragilis* NCTC 9343, essentially free from RNA and proteins, was further purified by hydrophobic-interaction gel chromatography using Octyl-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). A column (2.6 by 90 cm) was charged with 200 mg of LPS, which had been dissolved in distilled water, and eluted with 2 bed volumes of distilled water. LPS attached to the gel was eluted with 50% (vol/vol) aqueous ethanol, and fractions were monitored for carbohydrates by phenol-sulphuric acid (3). Fractions containing carbohydrates were pooled and lyophilized after reduction of the volume by evaporation.

Authentic fatty acids. Standard normal, as well as branched (iso- and anteiso-), fatty acids were purchased from Atlanta (Heidelberg). The preparation of racemic 3-hydroxydodecanoic, 3-hydroxytetradecanoic, and 3-hydroxyhexadecanoic acids, as well as the isolation of the corresponding D and L isomers, has been described (21).

Modification of fatty acids. Fatty acids were liberated from the LPS as described (7). Free fatty acids were carbomethylated with diazomethane in diethyl ether (22). The L-phenylethylamides of 3-methoxy fatty acids used for configuration analysis of 3-hydroxy fatty acids were prepared as described previously (21).

GLC. Methyl esters of fatty acids were separated isothermally (150°C) on a WCOT nonpolar glass capillary column (CP Sil 5, polydimethylsiloxane, 25 m; Chrompack, Berlin) with H_2 as carrier gas (0.7 bar).

The D and L forms of 3-methoxy fatty acid L-phenylethylamides were separated isothermally (210°C) on a WCOT OV 101 (polydimethylsiloxane) glass capillary column (25 m; Weecke, Mühlheim) with H_2 as carrier gas (0.7 bar). The columns were fitted to a Varian gas chromatograph (model 3700), which was equipped with flame ionization detectors and which was connected to a Hewlett-Packard integrator (model 3380 A). For quantitative analyses, heptadecanoic acid (methyl ester) was used as an internal standard.

Mass spectrometry. A Finnigan mass spectrometer (model 3200) with a CP Sil 5 glass capillary column (25 m) was used. Helium served as carrier gas, and gas-liquid chromatography (GLC) runs were performed with a temperature program (180 to 210°C, 1°C/min). Other conditions of mass spectrometric analyses were as described (11).

RESULTS

Qualitative analysis of fatty acids. For liberation of total fatty acids, LPS (*B. fragilis* NCTC 9343, 2 mg) was first treated with acid (4 N HCl, 4 h, 100°C) and then with alkali (1 N NaOH, 1 h, 100°C). The extracted free fatty acids were converted to methyl esters and analyzed by GLC. The fatty acid methyl ester pattern obtained is shown in Fig. 1. With the aid of authentic standard fatty acids (relative retention times) and mass spectrometry (specific fragments), the fatty acids corresponding to most of the peaks observed could be identified. Table 1 summarizes relative retention times (GLC) and characteristic fragments (mass spectrometry) of the individual peaks.

The prominent peaks corresponded to 13-methyl-tetradecanoic (peak 3), 3-hydroxypentadecanoic (peak 10), 3-hydroxyhexadecanoic (peak 13), 3-hydroxy-15-methyl-hexadecanoic (peak 14), and 3-hydroxyheptadecanoic acid methyl esters (peak 15). The minor peaks were identified as dodecanoic (peak 1), tetradecanoic

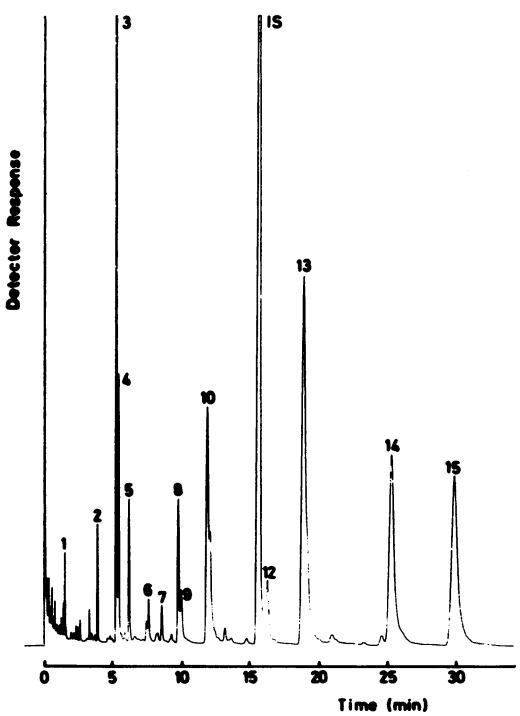

FIG. 1. *GL chromatogram of fatty acids of LPS from B. fragilis NCTC 9343 obtained on a CP Sil 5 capillary column (150°C). (Peak IS represents heptadecanoic acid methyl ester added as an internal standard).*

TABLE 1. *Characterization of fatty acids present in B. fragilis (NCTC 9343) LPS*

Peak no.[a]	Fatty acid methyl ester	GLC relative retention time[b]	Mass spectrometry (*m/e*)	
			Base peak	Characteristic ions
1	12:0	0.47	74	214 (M)
2	14:0	1.00	74	242 (M)
3	13-Me-14:0	1.30	74	256 (M), M-31 > M-29
4	12-Me-14:0	1.34	74	256 (M), 213 (M-43)
5	15:0	1.51	74	256 (M), 213 (M-43)
7	16:1	2.06	55	268 (M), 236 (M-32)
8	16:0	2.33	74	270 (M), 227 (M-43)
6	3-OH-14:0	1.84	103	208 (M-50)
10	3-OH-15:0	2.79	103	222 (M-50)
13	3-OH-16:0	4.35	103	236 (M-50)
14	3-OH-15-Me-16:0	5.77	103	250 (M-50)
15	3-OH-17:0	6.79	103	250 (M-50)

[a] Cf. Fig. 1; peak 9 corresponds to phthalic acid ester. Peaks 11 and 12 could not be identified.
[b] Relative retention times (t_R) are based on tetradecanoic acid methyl ester (14:0, t_R = 1.00).

(peak 2), 12-methyl-tetradecanoic (peak 4), pentadecanoic (peak 5), 3-hydroxytetradecanoic (peak 6), hexadecenoic (peak 7), and hexadecanoic acid methyl esters (peak 8). Peak 9 was shown to represent contaminating phthalic acid ester as indicated by the abundant fragment at m/e = 149 in the mass spectrum. Peaks 11 and 12 could not be characterized. The nature of the other peaks which were present only in trace amounts is indicated in Table 1.

Quantitative analysis of fatty acids. The amount of individual fatty acids present in LPS from *B. fragilis* was calculated from gas-liquid chromatograms with heptadecanoic acid as an internal standard (Fig. 1). As shown in Table 2, 13-methyl-tetradecanoic acid (0.144 μmol/mg), 3-hydroxypentadecanoic (0.067 μmol/mg), 3-hydroxyhexadecanoic (0.127 μmol/mg), 3-hydroxy-15-methyl-hexadecanoic (0.065 μmol/mg), and 3-hydroxyheptadecanoic (0.075 μmol/mg) acids predominated. In total, 0.54 μmol (14.8% wt) of fatty acids per mg were present in the LPS. Of the total fatty acids, 63% were hydroxy fatty acids.

Differentiation between ester- and amide-bound fatty acids. In LPS preparations of different bacterial origins, fatty acids are, in general, present in both ester and amide linkage (6, 23, 31). To test whether this was also true for *B. fragilis*, LPS was first subjected to alkaline methanolysis ($NaOCH_3$). This treatment is known to selectively release ester-bound fatty acids in the form of methyl esters (transesterification). Fatty acid esters were extracted from the methanolysate and analyzed by GLC (CP Sil 5, 150°C). It was found that all non-hydroxylated fatty acids present in LPS are exclusively ester bound (Table 2). Also, a part of 3-hydroxypentadecanoic (0.064 μmol/mg), 3-hydroxyhexadecanoic (0.088 μmol/mg), and 3-hydroxyheptadecanoic (0.034 μmol/mg) acids was found to be involved in ester linkage (Table 2). In total, therefore, 0.39 μmol (72%) of total fatty acids per mg was present as O-acyl residues.

TABLE 2. *Quantitative analysis of total and ester- and amide-bound fatty acids present in B. fragilis (NCTC 9343) LPS*

Fatty acid methyl ester[a]	Type of linkage[b]	Amt of fatty acids (μmol/mg of LPS)		
		Total[c]	Ester bound[c]	Amide bound[c]
12:0	E	0.001	0.001	ND[d]
14:0	E	0.005	0.004	ND
13-Me-14:0	E	0.144	0.147	0.003
12-Me-14:0	E	0.021	0.022	ND
15:0	E	0.012	0.012	ND
16:0	E	0.02	0.017	0.01
3-OH-14:0	E	0.004	0.004	ND
3-OH-15:0	EA	0.067[e]	0.064[e]	0.016
3-OH-16:0	EA	0.127	0.088	0.057
3-OH-15-Me-16:0	A	0.065	0.002	0.063
3-OH-17:0	EA	0.075	0.034	0.042

[a] Trace amounts (<0.001 μmol/mg) of 11-Me-13:0, 14-Me-15:0, 15-Me-16:0, 18:0, 16:1, 18:1, 3-OH-12:0, and 3-OH-11-Me-12:0 were present.
[b] Abbreviations: E, exclusively ester bound; EA, ester and amide bound; A, exclusively amide bound.
[c] Percentage of total: total, 14.8%; ester bound, 10.6%; amide bound, ~5%.
[d] ND, Not detectable.
[e] Double peak.

It is worthwhile to note that 3-methoxy fatty acid methyl esters could not be detected in the methanolysate. This shows that the 3-hydroxyl group of ester-bound 3-hydroxy fatty acids was not substituted (6, 22, 24).

Amide-bound fatty acids were liberated from the de-O-acylated preparation (obtained by alkaline methanolysis) by acidic and subsequent

alkaline hydrolysis, converted to methyl esters, and analyzed by GLC (Table 2). The pattern obtained showed small amounts of 3-hydroxypentadecanoic (0.016 μmol/mg) and larger amounts of 3-hydroxyhexadecanoic (0.057 μmol/mg), 3-hydroxy-15-methyl-hexadecanoic (0.063 μmol/mg), and 3-hydroxyheptadecanoic acid methyl esters (0.042 μmol/mg). This shows that 3-hydroxy-15-methyl-hexadecanoic acid is exclusively amide bound and that the other 3-hydroxy fatty acids are involved in both ester and amide linkage (Table 2).

Configuration analysis of 3-hydroxy fatty acids. Fatty acids were liberated from LPS by acid and subsequent alkali treatment. The 3-hydroxy fatty acids present in the hydrolysate were converted to diastereomeric 3-methoxy fatty acid L-phenylethylamides and subjected to GLC analysis (OV 101, 210°C). Five peaks were observed, the relative retention times of which were compared with those of authentic D- and L-3-methoxy fatty acid L-phenylethylamides or to the relative retention time values obtained by graphical extrapolation (Table 3). It was found that the peaks seen corresponded to the L-phenylethylamides of D-3-methoxytetradecanoic, D-3-methoxypentadecanoic, D-3-methoxyhexadecanoic, D-3-methoxy-15-methyl-hexadecanoic, and D-3-methoxyheptadecanoic acids. Therefore, all 3-hydroxy fatty acids present in *B. fragilis* LPS possess the D-configuration.

TABLE 3. *Relative retention times of diastereomeric 3-methoxy-L-phenylethylamide derivatives of standard and B. fragilis-derived 3-hydroxy fatty acids*

L-Phenylethylamide	Relative retention time[a] of:	
	Standard	Bacterial sample
D-3-OCH_3-12:0	0.52	
L-3-OCH_3-12:0	0.58	
D-3-OCH_3-14:0	1.00	1.00
L-3-OCH_3-14:0	1.08	
D-3-OCH_3-15:0	1.36[b]	1.36
L-3-OCH_3-15:0	1.48[b]	
D-3-OCH_3-16:0	1.86	1.85
L-3-OCH_3-16:0	2.02	
D-3-OCH_3-17:0	2.53[b]	2.52
L-3-OCH_3-17:0	2.78[b]	
D-3-OCH_3-9-Me-10:0	0.36	
L-3-OCH_3-9-Me-10:0	0.39	
D-3-OCH_3-11-Me-12:0	0.66	
L-3-OCH_3-11-Me-12:0	0.72	
D-3-OCH_3-15-Me-16:0	2.25[b]	2.25
L-3-OCH_3-15-Me-16:0	2.50[b]	

[a] Relative retention times (t_R) are based on D-3-methoxytetradecanoic acid-L-phenylethylamide (t_R = 1.00).

[b] Values were obtained by graphical extrapolation.

Fatty acid pattern of LPS from other *Bacteroides* strains. Table 4 gives the results of fatty acid analyses on LPS purified from *B. fragilis* NCTC 9343 and strain E-323, as well as on LPS (crude phenol-water extracts) from four other *Bacteroides* species. Compared to *B. fragilis*, *B. thetaiotaomicron* showed a similar fatty acid composition. The pattern obtained with the other species tested (*B. ovatus*, *B. distasonis*, *B. vulgatus*) differed from that of *B. fragilis* in that larger amounts of anteisobranched pentadecanoic acid (instead of the isobranched isomer) were present. In addition, in these cases no or only minor amounts of 3-hydroxyheptadecanoic acid were found. With regard to other fatty acids, the different *Bacteroides* species exhibited a qualitatively comparable fatty acid spectrum.

DISCUSSION

The LPS of *B. fragilis* consists, like LPS preparations of other gram-negative bacteria, of a polysaccharide and a lipid portion. Previous chemical investigations into the polysaccharide component have shown that this LPS portion is distinct from enterobacterial LPS in that it lacks 2-keto-3-deoxyoctonate and L-glycero-D-mannoheptose (10, 12). These core sugars are also absent from the LPS of *Bacteroides melaninogenicus* (8) and *Bacteroides asaccharolyticus* (17). Little was known, however, about the composition of the lipid component of *B. fragilis* LPS. In the present paper, the constituent fatty acids were qualitatively and quantitatively analyzed. The major fatty acids were identified as isobranched pentadecanoic (13-methyl-tetradecanoic), D-3-hydroxypentadecanoic, D-3-hydroxyhexadecanoic, isobranched D-3-hydroxyheptadecanoic (D-3-hydroxy-15-methyl-hexadecanoic), and D-3-hydroxyheptadecanoic acids. D-3-Hydroxytetradecanoic acid, which represents a major component of enterobacterial lipid A, was only present in minor amounts. The patterns were similar in the other *Bacteroides* species tested, except that in *B. ovatus*, *B. distasonis*, and *B. vulgatus*, larger amounts of anteisobranched pentadecanoic and no or smaller amounts of 3-hydroxyheptadecanoic acid were present. The lipid portion of *B. fragilis* LPS, therefore, is characterized by the presence of branched fatty acids (43%) and a range of D-3-hydroxy fatty acids (63%).

It is noteworthy that in one study on *B. fragilis* endotoxin, 3-hydroxy fatty acids were not found (1). In this report, however, a number of fatty acids could not be identified which, according to their retention times (GLC), may well

TABLE 4. *Fatty acid composition of LPS from different Bacteroides species*

Fatty acid	LPS fatty acid composition (mol %) of:					
	B. fragilis NCTC 9343	*B. fragilis* E-323	*B. thetaiotaomicron* NCTC 10582	*B. ovatus* ATCC 8483	*B. distasonis* ATCC 8503	*B. vulgatus* ATCC 8482
12:0	0.2	ND[a]	1	2	ND	3
14:0	1	3	2	5	5	8
13-Me-14:0	27	17	20	3	3	3
12-Me-14:0	4	4	4	13	12	11
15:0	2	3	5	5	7	7
16:1	Trace	Trace	3	7	7	10
16:0	4	9	8	24	26	30
3-OH-14:0	1	1	2	1	ND	2
3-OH-15:0[b]	12	11	16	19	19	10
3-OH-16:0	23	32	19	9	7	7
3-OH-15-Me-16:0	12	15	13	10	14	9
3-OH-17:0	14	7	7	2	ND	ND

[a] ND, Not detectable.
[b] Double peak.

represent long-chain hydroxylated fatty acids.

The cellular fatty acid pattern of most *Bacteroides* species is characterized by the presence of 3-hydroxylated and branched or odd-numbered acids (4, 18). Thus, the main cellular fatty acids of *B. fragilis* are anteisobranched pentadecanoic acid and isobranched 3-hydroxyheptadecanoic acid. 3-Hydroxyhexadecanoic acid is also found in substantial amounts. These fatty acids were shown to be present in sphingolipids of *B. fragilis* (5) and other *Bacteroides* species (14, 18, 26, 30). The present study shows that they also occur as constituents of *Bacteroides* LPS.

Isobranched fatty acids have previously been identified in LPS or other (bound) lipids of only a few bacterial groups. These include *Pseudomonas rubescens* (32), *Pseudomonas maltophilia* (19), *Coxiella burneti* (2), *Xanthomonas sinensis* (24), and *Myxococcus fulvus* (25). In these cases, both non-hydroxylated and 3-hydroxylated fatty acids are isobranched.

In *B. fragilis* LPS five distinct 3-hydroxy fatty acids are present. Such a range of hydroxy fatty acids has previously been found in the LPS of *Xanthomonas* (24), *P. maltophilia* (19), *Brucella abortus* (13), and some other bacteria (for reviews, see references 6, 31). Among the 3-hydroxy fatty acids of *B. fragilis* LPS, we also found 3-hydroxytetradecanoic acid, which represents the predominant fatty acid in enterobacterial LPS. In *B. fragilis*, however, this fatty acid was only a minor component, and in *B. melaninogenicus* LPS it is absent (17).

All 3-hydroxy fatty acids present in *B. fragilis* LPS were found to belong to the D-series. This is in accordance with previous stereochemical investigations which had shown that 3-hydroxy fatty acids in the LPS of distinct bacterial groups possess, regardless of chain length, branching, or type of linkage, the D configuration.

In general, fatty acids are present in lipid A in both ester and amide linkages. This is also the case in *B. fragilis* LPS. Here, as in other LPS, non-hydroxylated fatty acids (mainly isobranched pentadecanoic acid) are exclusively ester linked. A part of the 3-hydroxy fatty acids present is also involved in ester linkage. However, *N*-acyl residues are exclusively represented by D-3-hydroxy fatty acids, isobranched D-3-hydroxyheptadecanoic acid being exclusively amide bound.

The general principles governing the fatty acid composition of *Bacteroides* LPS (presence of ester- and amide-bound acyl residues, notably the presence of amide-bound D-3-hydroxy fatty acids) are identical to those observed in the LPS of most other gram-negative bacteria studied so far (23). However, the fatty acid pattern of *B. fragilis* LPS differs from that of the majority of other gram-negative organisms in that isobranched fatty acids and a range of D-3-hydroxylated fatty acids predominate. Studies are now in progress to elucidate further details of the *Bacteroides* lipid A structure, and it is hoped that these investigations may also allow insight

into the problem of whether the unusual fatty acid composition of *B. fragilis* LPS is related to its low endotoxic activity.

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